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Colonization of Reproductive Organs and Internal Contamination of Eggs After Experimental Infection of Laying Hens with *Salmonella heidelberg* and *Salmonella enteritidis*

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SUMMARY. Internal contamination of eggs laid by hens infected with *Salmonella enteritidis* has been a prominent international public health issue since the mid-1980s. Considerable resources have been committed to detecting and controlling *S. enteritidis* infections in commercial laying flocks. Recently, the Centers for Disease Control and Prevention also reported a significant association between eggs or egg-containing foods and *S. heidelberg* infections in humans. The present study sought to determine whether several *S. heidelberg* isolates obtained from egg-associated human disease outbreaks were able to colonize reproductive tissues and be deposited inside eggs laid by experimentally infected hens in a manner similar to the previously documented behavior of *S. enteritidis*. In two trials, groups of laying hens were orally inoculated with large doses of four *S. heidelberg* strains and an *S. enteritidis* strain that consistently caused egg contamination in previous studies. All five *Salmonella* strains (of both serotypes) colonized the intestinal tracts and invaded the livers, spleens, ovaries, and oviducts of inoculated hens, with no significant differences observed between the strains for any of these parameters. All four *S. heidelberg* strains were recovered from the interior liquid contents of eggs laid by infected hens, although at lower frequencies (between 1.1% and 4.5%) than the *S. enteritidis* strain (7.0%).

RESUMEN. Colonización de los órganos reproductivos y contaminación interna de los huevos después de la infección experimental de ponedoras comerciales con *Salmonella heidelberg* y *Salmonella enteritidis*.

La contaminación interna en huevos obtenidos a partir de gallinas contaminadas con *Salmonella enteritidis* ha sido un punto de preocupación prominente en el área de salud pública a nivel internacional desde mediados de la década de 1980. Durante este periodo se han asignado cuantiosos recursos para la detección y control de las infecciones por *Salmonella enteritidis* en parvadas de ponedoras comerciales. Recientemente, los centros para el control y prevención de enfermedades han reportado que existe una asociación significativa entre los huevos y los alimentos que contienen huevo, y la infección por *S. heidelberg* en humanos. El presente estudio determinó si cepas de *S. heidelberg*, aisladas a partir de muestras tomadas en brotes de enfermedad asociados al consumo de huevos en humanos, eran capaces de colonizar el tracto reproductivo y ser transmitidas a los huevos en aves infectadas en forma experimental, al igual que como ha sido reportado en infecciones por *Salmonella enteritidis*. En dos experimentos diferentes, se inocularon grupos de ponedoras por la vía oral con dosis altas de inóculos de 4 cepas diferentes de *S. heidelberg* y una cepa de *S. enteritidis*, las cuales han causado en forma consistente contaminación de huevos en estudios previos. Las cinco cepas utilizadas fueron capaces de colonizar el tracto gastrointestinal de las aves y capaces de invadir los hígados, bazos, ovarios y oviductos de las aves inoculadas. No se observaron diferencias significativas en estos parámetros entre las cepas utilizadas en el estudio. La cuatro cepas de *S. heidelberg* fueron reaisladas a partir de muestras del contenido líquido de los huevos obtenidos a partir de las aves inoculadas, aunque en menor frecuencia (entre 1.1% y 4.5%) en comparación con la cepa de *S. enteritidis*.

Key words: *Salmonella heidelberg*, *Salmonella enteritidis*, chickens, reproductive organs, egg contamination

Abbreviations: BG = brilliant green; TS = tryptone soya

The international significance of contaminated eggs in the transmission of *Salmonella enterica* serovar *enteritidis* (*S. enteritidis*) infection to humans has been a focus for discussion, research, and regulatory activity during the past two decades (1,7). Eggs containing this pathogen in their edible liquid contents have been produced by both experimentally and naturally infected hens (14,19,28,29). Reducing the occurrence of *S. enteritidis* infections in commercial laying flocks has become an important public health objective (26,42). A recent survey detected *S. enteritidis* in the laying house environments of approximately 7% of the sampled egg-producing flocks in the United States (46). Nevertheless, the overall incidence of *S. enteritidis* contamination in eggs has been estimated to be only about 0.005% (13).

The deposition of *S. enteritidis* within eggs seems to result from the colonization of reproductive organs, particularly the ovary and upper oviduct, in systemically infected hens (11,31,35,36). The site of *S. enteritidis* deposition in eggs (albumen or yolk) may be determined by the region of the hen's reproductive tract that is colonized (3,19,28,29). However, high frequencies of reproductive tissue colonization do not always lead to correspondingly high frequencies of egg contamination (2,34). Even after the administration of large oral doses of *S. enteritidis* to laying hens, egg contamination usually occurs at a low incidence (17,19,20,27) and typically involves small numbers of *S. enteritidis* cells (15,17,19,28,29). Although fecal shedding of *S. enteritidis* into the laying house environment has been a useful indicator of flock infection status, persistent intestinal colonization by *S. enteritidis* has not been a reliable predictor of the likelihood of systemic infection and egg contamination (16,19,27).

Despite the uniquely significant epidemiological association between *S. enteritidis* and eggs, other *Salmonella* serotypes have also been shown to be capable of colonizing reproductive tissues in chickens and sometimes reaching the contents of developing eggs (32,37,45). Eggs contaminated by *S. enterica* serovar *heidelberg* (*S. heidelberg*) have sometimes been implicated as food vehicles in human disease outbreaks (4). In a study conducted by the Centers for Disease Control and Prevention (10), approximately 23% of *S. heidelberg* outbreaks in the United States since 1973 were attributed to eggs or egg-containing foods (a similar proportion of outbreaks was associated with other poultry products). Another recent report from this same agency indicated that eating eggs prepared outside the home was the most significant risk factor identified in investigations of sporadic *S. heidelberg* infections (25). Responsible for at least 2000 culture-confirmed human illnesses annually, *S. heidelberg* was among the four most common *Salmonella* serotypes isolated from humans in both 2001 and 2002 (5,6,10).

In reports from diverse locations in the United States and Canada, *S. heidelberg* has consistently been among the *Salmonella* serotypes found most often in commercial egg-laying flocks (12,40,41,43) and has sometimes been detected in association with dirty or cracked egg shells (30,39). Like *S. enteritidis*, some *S. heidelberg* strains have exhibited a high degree of virulence in chicks (38,44). However, despite the mounting evidence for an epidemiological connection between *S. heidelberg* and eggs, little is known about whether strains of this serotype can be deposited inside developing eggs in a manner analogous to the better characterized behavior of *S. enteritidis*. Therefore, the objective of the present study was to determine whether several *S. heidelberg* strains, obtained from human disease cases associated with eggs or egg-containing foods, would colonize reproductive tissues and be deposited in the liquid contents of eggs laid by experimentally infected hens.

MATERIALS AND METHODS

Experimental infection of laying hens. In each of two trials, 72 laying hens obtained from our laboratory's specific-pathogen-free flock of single-comb white leghorn chickens were distributed evenly among three separately housed groups in a disease-containment facility. The hens (28 and 33 wk old at the beginning of the first and second trials, respectively) were kept in individual laying cages and provided with water and pellet feed *ad libitum*.

The three groups of chickens in each trial were inoculated with different *Salmonella* strains. One group of hens in each trial received a phage type 13a *S. enteritidis* isolate (designated strain 6) that has been consistently associated with egg contamination by infected hens in previous experiments (18,19,20,21). The other two groups of hens in each trial were inoculated with *S. heidelberg* isolates (strains 4 and 11 in trial 1 and strains 1 and 5 in trial 2). These *S. heidelberg* strains (provided by Dr. B. Swaminathan, Centers for Disease Control, Atlanta, GA) were originally isolated from humans during disease outbreaks for which eggs were an implicated food source. Each hen was given a 1-ml oral dose containing approximately 1.5×10^9 of colony-forming units

of the appropriate *Salmonella* strain, prepared by incubation in tryptone soya (TS) broth (Oxoid Limited, Basingstoke, Hampshire, England) for 24 hr at 37 C.

Fecal samples. Immediately before inoculation and at 1, 2, and 3 wk postinoculation, sterile cotton swabs were used to collect samples of voided feces from polystyrene trays (food grade but not sterile) placed under each cage. These samples were transferred to 9 ml of tetrathionate broth (Oxoid) and incubated for 24 hr at 37 C. A 10-µl portion from each broth culture was then streaked onto brilliant green (BG) agar (Becton, Dickinson, and Co., Franklin Lakes, NJ) supplemented with 0.02 mg/ml of novobiocin (Sigma Chemical Co., St. Louis, MO) and incubated for 24 hr at 37 C. The identity of presumptive colonies of *S. enteritidis* or *S. heidelberg* was biochemically and serologically (48) confirmed.

Internal organ samples. At 7 days and 21 days after inoculation in each trial, six hens were randomly selected from each treatment group and humanely euthanatized to allow removal of internal tissues for bacteriologic culture. Portions (approximately 5–10 g) of the liver, spleen, ovary, oviduct, and ceca (including the ileocecal junction) from each hen were aseptically removed, transferred to 25 g of tetrathionate broth, and mixed by stomaching for 30 sec. Each broth culture was incubated for 40 hr at 37 C, and a 10-µl aliquot was then streaked onto BG agar plus novobiocin. After incubation of these plates for 24 hr at 37 C, typical *S. enteritidis* or *S. heidelberg* colonies were subjected to biochemical and serologic confirmation.

Egg contents samples. All eggs laid on the day before inoculation and during the first 22 days after inoculation were cultured to detect internal contamination with *Salmonella*. Eggshell surfaces were disinfected by dipping for 5 sec in 70% ethanol, and the shells were then broken against a sharp edge covered by sterile foil strips. The entire liquid contents of each egg were transferred to 50 ml of TS broth supplemented with 100 mg/l of ferrous sulfate (Sigma), mixed by vigorous shaking for 15 sec, and incubated for 24 hr at 37 C. A 1-ml portion of each incubated TS broth culture was transferred to 9 ml of Rappaport Vassiliadis broth (Oxoid) and incubated for 24 hr at 37 C. A 10-µl aliquot from each of these broth cultures was then streaked onto BG agar and incubated for 24 hr at 37 C. The identity of typical colonies of *S. enteritidis* or *S. heidelberg* was biochemically and serologically confirmed.

Statistical analysis. For each trial, significant differences ($P < 0.05$) between treatment groups in the mean frequency of recovery of *Salmonella* strains from voided feces, internal organs, or egg contents were determined by Kruskal-Wallis analysis of variance followed by Dunn multiple-comparison test. Data were analyzed with Instat biostatistics software (GraphPad Software, San Diego, CA).

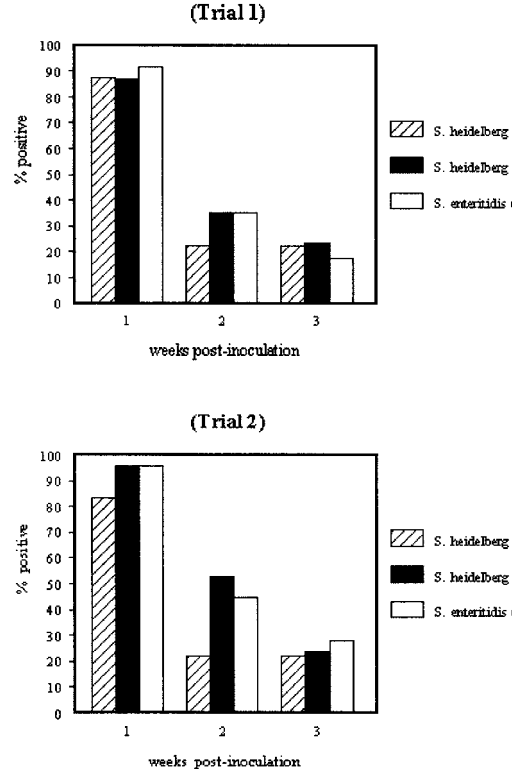

Fig. 1. Frequency of recovery of *S. heidelberg* and *S. enteritidis* strains from samples of voided feces after oral inoculation of laying hens in two trials ($n = 24$ per treatment group at 1 wk after inoculation, and $n = 18$ per group at 2 wk and 3 wk after inoculation).

RESULTS

Detection of *S. heidelberg* and *S. enteritidis* in fecal samples. All fecal samples collected before inoculation of the hens were negative for *Salmonella*. At 1 wk after inoculation, the frequencies of recovery of the various *Salmonella* strains from voided feces ranged from 83.3% to 95.8% (Fig. 1). By 3 wk after inoculation, the recovery of *S. heidelberg* or *S. enteritidis* strains from fecal samples had declined to a range of 17.7% to 27.8%. No significant differences between *Salmonella* strains in their frequencies of isolation from feces were observed in either trial.

Detection of *S. heidelberg* and *S. enteritidis* in internal organs. No significant differences between *Salmonella* strains in their frequencies of isolation from internal organs were observed in either trial (Table 1). Nearly all cecal samples were positive for *Salmonella* at 7 days after inoculation,

Table 1. Recovery of *S. heidelberg* and *S. enteritidis* strains from internal organs of orally inoculated laying hens.[A]

Salmonella strain	7 days after inoculation					21 days after inoculation				
	Liver	Spleen	Ovary	Oviduct	Cecum	Liver	Spleen	Ovary	Oviduct	Cecum
	Salmonella-positive/total									
Trial 1:										
S. heidelberg 1	5/6	5/6	1/6	2/6	6/6	0/6	1/6	1/6	0/6	3/6
S. heidelberg 5	6/6	6/6	4/6	4/6	6/6	0/6	2/6	0/6	0/6	4/6
S. enteritidis 6	6/6	6/6	3/6	1/6	6/6	0/6	1/6	0/6	0/6	3/6
Trial 2:										
S. heidelberg 4	3/6	5/6	1/6	2/6	5/6	0/6	1/6	0/6	0/6	3/6
S. heidelberg 11	6/6	6/6	4/6	3/6	6/6	0/6	2/6	0/6	0/6	3/6
S. enteritidis 6	6/6	6/6	2/6	2/6	6/6	0/6	1/6	1/6	0/6	3/6

[A] $n = 24$ hens per treatment group in each trial.

and approximately one half of the cecal samples from all groups were positive at 21 days. All isolates except *S. heidelberg* 4 were recovered from 83.3% or more of liver samples at 7 days after inoculation, but no liver samples were positive at 21 days. Similarly, at least 83.3% of spleen samples from all treatment groups were *Salmonella*-positive at 7 days after inoculation, but the corresponding recovery frequencies at 21 days ranged only from 16.7% to 33.3%. All *S. heidelberg* and *S. enteritidis* strains were found in both ovaries and oviducts at 7 days, at frequencies as high as 66.7% (*S. heidelberg* 11 in ovaries and *S. heidelberg* 5 in both reproductive tissues). However, at 21 days after inoculation, ovarian recoveries of *Salmonella* were infrequent (never exceeding 16.7% in any group), and no oviduct recoveries were made.

Detection of *S. heidelberg* and *S. enteritidis* in egg contents samples. All eggs collected before inoculation of the hens were negative for *Salmonella* in their liquid contents. All *S. heidelberg* and *S. enteritidis* strains used in this study were deposited inside eggs laid by infected hens (Table 2). The observed frequencies of internal contamination of eggs ranged from 1.11% for *S. heidelberg* 4 to 7.05% for *S. enteritidis* 6 (in trial 2). In trial 1, a significantly ($P < 0.05$) larger proportion of contaminated eggs was laid by the group infected with *S. enteritidis* 6 than by the group given *S. heidelberg* 1. Likewise, in trial 2, the frequency of egg contamination associated with *S. enteritidis* 6 was significantly ($P < 0.01$) higher than for *S. heidelberg* 4. No other significant differences between treatment groups were observed. The peak daily frequencies of egg contamination, for all *Salmonella* isolates in both trials, occurred between 7 and 10 days after inoculation. In both trials, the *S. enteritidis* 6 strain was deposited in eggs over a longer postinoculation interval than were any of the *S. heidelberg* strains.

DISCUSSION

The various *S. enteritidis* and *S. heidelberg* strains evaluated in the present study were associated with similar trends over time in the intestinal colonization of infected hens. All strains were found in the ceca and shed in the feces of nearly all inoculated birds at 1 wk after inoculation, but few of these hens were still shedding *Salmonella* in their feces by 3 wk after inoculation. Previous experiments have not established a dependable relationship between the persistence of fecal shedding and the production of eggs containing *S. enteritidis* (16,19,27). A large degree of similarity between the *S. enteritidis* and *S. heidelberg* strains was also observed in their frequencies of isolation from livers and spleens in the present study. Most of these samples were positive at 1 wk after inoculation, but only a small percentage were still positive at 3 wk after inoculation. The ability of *S. enteritidis* strains to invade the liver and spleen, although indicative of systemic infection that might also reach reproductive organs, has not always correlated with the frequency of deposition of the pathogen inside eggs in earlier studies (16).

All five *Salmonella* strains used in the current study were found in both ovaries and oviducts of some sampled hens at 7 days after inoculation. The colonization of reproductive tissues of chickens by serotypes other than *S. enteritidis*, particularly *S. heidelberg* and *S. typhimurium*, has also been de-

Table 2. Recovery of *S. heidelberg* and *S. enteritidis* strains from eggs laid by orally inoculated laying hens.[A]

Salmonella strain	*Salmonella*-positive eggs/total (%)	First and last contaminated eggs laid (days after inoculation)	Peak frequency of egg contamination attained (days after inoculation)
Trial 1:			
S. heidelberg 1	5/256 (1.95 %)[B]	10–21	10
S. heidelberg 5	11/244 (4.52%)[BC]	5–17	9
S. enteritidis 6	15/218 (6.88%)[C]	5–20	7–9
Trial 2:			
S. heidelberg 4	3/270 (1.11%)[B]	9–10	9
S. heidelberg 11	9/278 (3.24%)[BC]	5–11	8
S. enteritidis 6	21/298 (7.05%)[C]	3–21	10

[A]The entire liquid contents of all eggs laid for 22 days after inoculation ($n = 24$ hens per treatment group in each trial) were cultured.

[BC]Egg contamination frequencies within a trial are significantly ($P < 0.05$) different if they share no common superscripts.

scribed in several previous reports (32,37,45). The *S. enteritidis* 6 strain has consistently induced the production of internally contaminated eggs in previous oral infection experiments (18,19,20,21), but it was not recovered from reproductive organs in the present study at a higher frequency than were the four *S. heidelberg* strains. Although *S. enteritidis* colonization of the ovaries and oviducts of infected hens has been extensively documented as a necessary step in the pathway that leads to bacterial deposition in eggs (11,31,35,36), the presence of this pathogen in reproductive tissues is not sufficient to guarantee that egg contamination will occur at a high frequency (2,34). All four *S. heidelberg* strains in the present study were deposited inside eggs, although two of these strains contaminated eggs at very small frequencies (<2%), and none of the *S. heidelberg* strains were isolated from eggs as often as the *S. enteritidis* 6 strain. A relatively small incidence of egg contamination has been a common feature of most experimental infection studies, even when hens received large oral doses of *S. enteritidis* (17,19,20,27). Naturally occurring infections are likely to involve exposure to much smaller doses of *Salmonella*, and the observed incidence of egg contamination in commercial laying flocks has been correspondingly much lower than is typically reported in experimental infection studies (13,26,28,29).

The mechanisms by which *S. enteritidis* colonizes reproductive tissues of chickens and is deposited in eggs remain subjects for ongoing inquiry and research. Strains of *S. enteritidis* can differ considerably in the ability to contaminate eggs (19,21). Phenotypic attributes such as the production of high molecular mass lipopolysaccharide and growth to high cell densities, especially when expressed together in a complementary manner by different bacterial subpopulations, have been linked to egg contamination by *S. enteritidis* strains (17,22,23,24). Environmental conditions (including pH, temperature, and growth in chicken tissues) can affect the expression of *S. enteritidis* virulence factors such as flagella, fimbria, outer membrane proteins, and iron uptake systems (8,9,33,47). Repeated *in vivo* passage of an *S. enteritidis* strain through reproductive tissues of chickens has been shown to increase the frequency of egg contamination caused by this strain (18).

Because *S. heidelberg* is one of the *Salmonella* serotypes isolated most often in egg-laying flocks (12,40,41,43), recent reports of a strong association between eggs and human illnesses caused by *S. heidelberg* have increased concerns about whether this organism is emerging as an important egg-transmitted pathogen (10,25). As demonstrated by the present experiment, some strains of *S. heidelberg* can indeed colonize the reproductive tract of chickens and induce the production of internally contaminated eggs. However, all four *S. heidelberg* strains used in the present study were originally isolated from human patients in outbreaks that were attributed to eggs or egg-containing foods. The overall extent to which the ability to cause egg contamination is distributed among all strains of this serotype is not yet known. Further investigation to compare the mechanisms by which *S. heidelberg* and *S. enteritidis* are deposited in eggs should provide both a clearer understanding of how

S. enteritidis has become such a significant public health risk and an assessment of whether *S. heidelberg* might pose a similar threat.

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